Biotechnology Advances xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Acetic acid bacteria: A group of bacteria with versatile biotechnological applications

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ARTICLE INFO

Available online xxxx

Keywords:
Acetic acid bacteria
Biotechnology
Thermotolerant
Oxidative fermentation
Vinegar

ABSTRACT

Acetic acid bacteria are gram-negative obligate aerobic bacteria assigned to the family *Acetobacteraceae* of *Alphaproteobacteria*. They are members of the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, *Tanticharoenia*, *Ameyamaea*, *Neokomagataea*, and *Komagataeibacter*. Many strains of *Acetobacter* and *Komagataeibacter* have been known to possess high acetic acid fermentation ability as well as the acetic acid and ethanol resistance, which are considered to be useful features for industrial production of acetic acid and vinegar, the commercial product. On the other hand, *Gluconobacter* strains have the ability to perform oxidative fermentation of various sugars, sugar alcohols, and sugar acids leading to the formation of several valuable products. Thermotolerant strains of acetic acid bacteria were isolated in order to serve as the new strains of choice for industrial fermentations, in which the cooling costs for maintaining optimum growth and production temperature in the fermentation vessels could be significantly reduced. Genetic modifications by adaptation and genetic engineering were also applied to improve their properties, such as productivity and heat resistance.

Contents

Abbreviations: 25DKGA, 2,5-diketo-D-gluconic acid; 2KGA, 2-keto-D-gluconic acid; 4KAB, 4-keto-D-arabonate; 4KAR, 4-keto-D-arabinose; 4KRB, 4-keto-D-ribose; 4KRN, 4-keto-D-ribonate; 5KGA, 5-keto-D-gluconic acid; AAB, acetic acid bacteria; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CAD, cyclic alcohol dehydrogenase; CPS, capsular polysaccharide; DQA, 3-dehydroquinate; DQD, 3-dehydroquinate dehydratase; DSA, 3-dehydroshikimate; DSD, 3-dehydroshikimate dehydratase; EPS, extracellular polysaccharide; FAD-GADH, FAD-dependent D-gluconate dehydrogenase; FAD-SLDH, FAD-dependent D-sorbitol dehydrogenase; GA, gluconic acid; PCA, protocatechuic acid; PQQ, pyrroloquinoline quinone; PQQ-ADH, PQQ-dependent alcohol dehydrogenase; PQQ-GDH, PQQ-dependent D-glucose dehydrogenase; PQQ-GLDH, PQQ-dependent glycerol dehydrogenase; QDH, quinate dehydrogenase

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http://dx.doi.org/10.1016/j.biotechadv.2014.12.001
0734-9750/

Introduction

Acetic acid bacteria (AAB) naturally produce acetic acid from ethanol. They are obligate aerobic bacteria widely spread in sugary or acidic substances in nature or in alcoholic beverages (Bartowsky and Henschke, 2008; Gupta et al., 2001; Mamlouk and Gullo, 2013; Moonmangmee et al., 2000; Raspor and Goranovič, 2008; Sengun and Karabiyikli, 2011). There are several new AAB isolated from different sources every year and it is likely that AAB can also be found elsewhere, not only in the usual sources where AAB are abundant. Isolation of AAB from natural habitats requires an enrichment of AAB in the presence of ethanol, sugars or sugar alcohols as carbon sources (Carr, 1968; Cirigliano, 1982; González et al., 2006).

AAB are known to have unique fermentation ability, so called "oxidative fermentation", which is a process of incomplete oxidation where the substrates are oxidized by membrane-bound dehydrogenases and the oxidized products are then released to culture medium (see Fig. 1). The electrons are abstracted from the substrate by a cofactor of the specific enzyme, and then transferred to the terminal oxidase via respiratory ubiquinone (Adachi et al., 2007; Mamlouk and Gullo, 2013; Matsushita et al., 1994; Matsutani et al., 2014; Miura et al., 2013; Richhardt et al., 2013). By the nature of oxidative fermentation, O_2 is constantly required; thus O_2 availability could severely affect the fermentation rate and thus productivity (Gullo et al., 2014; Qi et al., 2013; Schlepütz et al., 2013). The oxidative fermentation is a very important trait of AAB as the release of corresponding oxidized products to culture medium provides simple downstream processing. The most typical example of oxidative fermentation is the acetic acid production from ethanol. Ethanol is oxidized to acetaldehyde by a membrane-bound pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) and then acetaldehyde is further oxidized to acetic acid by a membrane-bound aldehyde dehydrogenase (ALDH). Upon oxidation, acetic acid accumulates in the culture medium. Among several genera of AAB, *Acetobacter* spp. and *Komagataeibacter* (formerly *Gluconacetobacter*; see Genera and species of AAB) spp. are most often used due to their high resistance to both ethanol and acetic acid that is very important for industrial vinegar production (Gullo et al., 2014). They are also capable of producing polysaccharides to help them floating on medium surface.

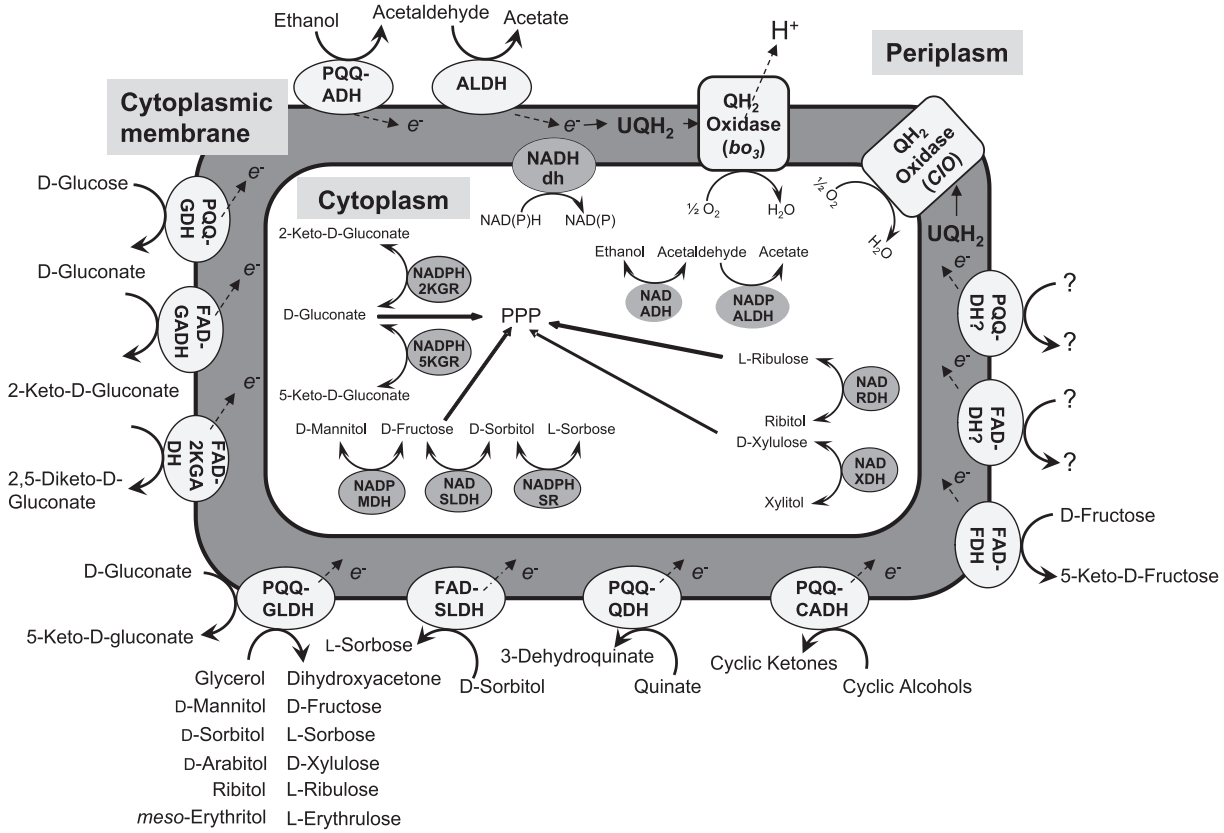

Fig. 1. Oxidative fermentation overview of acetic acid bacteria (adapted from Adachi et al., 2007; Prust et al., 2005). Membrane-bound primary dehydrogenases and some soluble enzymes known to be involved in production and assimilation of various compounds are illustrated. Membrane-bound dehydrogenases are responsible for product formation, whereas the enzymes in cytoplasm play a role in product assimilation. Note: D-glucono-δ-lactone can be converted to D-gluconate spontaneously or by a membrane-bound gluconolactonase (not shown it this figure). PQQ-ADH: PQQ-dependent alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; PQQ-GDH: PQQ-dependent D-glucose dehydrogenase; PQQ-GLDH: PQQ-dependent glycerol dehydrogenase; FAD-GADH: FAD-dependent D-gluconate dehydrogenase; FAD-SLDH: FAD-dependent D-sorbitol dehydrogenase; FAD-FDH: FAD-dependent D-fructose dehydrogenase; FAD-2KGA-DH: FAD-dependent 2-keto-D-gluconate dehydrogenase; NADH-dh: NADH dehydrogenase; QH_2 Oxidase (bo_3): cytochrome bo_3 quinol oxidase; QH_2 Oxidase (*CIO*): cyanide-insensitive quinol oxidase; 2KGR: 2-keto-D-gluconate reductase; 5KGR: 5-keto-D-gluconate reductase; ADH: alcohol dehydrogenase; RDH: ribitol dehydrogenase; XDH: xylitol dehydrogenase; SLDH: sorbitol dehydrogenase; MDH: mannitol dehydrogenase; QDH: quinate dehydrogenase; CADH: cyclic alcohol dehydrogenase; SR: sorbose reductase; UQH_2: ubiquinol, reduced form of ubiquinone; and PPP: pentose phosphate pathway.

Please cite this article as: Saichana N, et al, Acetic acid bacteria: A group of bacteria with versatile biotechnological applications, Biotechnol Adv (2014), http://dx.doi.org/10.1016/j.biotechadv.2014.12.001

These polysaccharides also support the uptake of O_2 on the medium surface and protect against various stresses, such as high acidity and high temperature (Deeraksa et al., 2005, 2006; Kanchanarach et al., 2010a). On the other hand, *Gluconobacter* spp. have been used to produce several valuable compounds, such as L-sorbose, 2-keto-D-gluconic acid (2KGA), 5-keto-D-gluconic acid (5KGA), dihydroxyacetone, and some cyclic ketones (Adachi et al., 2003b; Gupta et al., 2001). Due to the enantio- and regioselectivities of enzyme systems in *Gluconobacter*, they have been used for biotransformations of some compounds to prepare the products with desirable conformations, such as for enantioselective oxidation of chiral and prochiral alcohol and diols. For example, *Gluconobacter* spp. can be used for biotransformations of *N*-(2-hydroxyethyl) glucamine to produce 6-(2-hydroxyethyl) amino-6-deoxy-α-L-sorbofuranose, which is the key intermediate for synthesis of miglitol, a drug used for type II diabetes treatment (Keliang and Dongzhi, 2006). (*S*)-2-methylbutanoic acid, an aroma compound found in fruits and used in the food industry, has also been reported to be produced by *Gluconobacter* from 2-methylbutanol (Keliang and Dongzhi, 2006).

Although AAB are valuable biocatalysts useful in many applications, they are also recognized as spoiling bacteria in other processes such as in alcoholic beverage production, where acetic acid fermentation is unfavorable (Bartowsky and Henschke, 2008; Mamlouk and Gullo, 2013; Sengun and Karabiyikli, 2011). While most AAB have been considered safe as food processing bacteria, some strains have been reported to associate with human infections as emerging opportunistic pathogens. The infection by AAB (*Asaia bogorensis*) has been first reported in 2004 in a patient with a peritoneal dialysis catheter (Snyder et al., 2004). Another AAB, *Granulibacter bethesdensis*, was the cause of infection in patients suffering from chronic granulomatous disease (Greenberg et al., 2006a, 2006b). The symptoms of bacteremia caused by infection and colonization with AAB are rarely, but increasingly, reported in patients with underlying chronic diseases such as cystic fibrosis and in patients with indwelling devices including patients with history of intravenous drug abuse (Alauzet et al., 2010). The human pathogenic AAB known to date are *As. bogorensis* (Snyder et al., 2004), *Asaia lannaensis* (Abdel-Haq et al., 2009; Juretschko et al., 2010), *Acetobacter cibinongensis* (Gouby et al., 2007), *Gr. bethesdensis* (Greenberg et al., 2006b; López et al., 2008), and *Gluconobacter* spp. (Alauzet et al., 2010; Bassetti et al., 2013).

In this review, we describe the recent developments in oxidative fermentation by AAB in their biotechnological aspects. The contents include new interesting traits of some thermotolerant AAB and developments of expression systems for their efficient application.

Genera and species of AAB

In the past decades, classification of AAB has been reviewed and reclassification was done throughout the genera (Cleenwerck and De Vos, 2008; Yamada and Yukphan, 2008). AAB are gram-negative obligate aerobic bacteria assigned to the family *Acetobacteraceae* of *Alphaproteobacteria*. In 2008, Yamada and Yukphan classified AAB in ten genera: *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, and *Granulibacter*. The genus *Frateuria* was recognized as a pseudacetic acid bacterium, belonging to a group of *Gammaproteobacteria* (Yamada and Yukphan, 2008). Within the above mentioned genera of AAB, *Acetobacter* is a unique genus with ubiquinone-9 as its major respiratory quinone whereas other 9 genera have ubiquinone-10. On the other hand, the genus *Frateuria* has a unique ubiquinone-8 as the major respiratory quinone.

New genus and species of AAB, *Tanticharoenia sakaeratensis*, comprising type strain AC37^T (= BCC 15772^T = NBRC 103193^T) and strains AC38 and AC39, were proposed by Yukphan et al. in 2008. These new AAB did not oxidize acetate or lactate, but grew on 30% D-glucose. The 12th genus, *Ameyamaea*, was proposed when two new isolates, namely AC04 and AC05, of AAB were isolated from flowers of red ginger in Chiang Mai, Thailand (Yukphan et al., 2009). These strains were assigned as *Ameyamaea chaiangmaiensis*, the strain AC04^T (= BCC 15744^T, = NBRC 103196^T) being the type strain. They have polar flagella and show fast acetate oxidation rate equal to the type strain of *Acetobacter aceti*, an acetic acid producer. The 13th genus of AAB has been proposed with two new species *Neokomagataea thailandica* AH11^T (= BCC 25710^T, = NBRC 106555^T) and *Neokomagataea tanensis* AH13^T (= BCC 25711^T, = NBRC 106556^T) (Yukphan et al., 2011). These species were not motile and did not oxidize acetate or lactate. They were not osmophilic, but osmotolerant, and no growth was observed in 0.35% acetic acid. With new classification methodologies of bacteria and the growing information on AAB in recent years, Yamada et al. (2012a; 2012b) proposed the 14th genus named *Komagataeibacter* dividing from the genus *Gluconacetobacter*. The genus *Gluconacetobacter* has been divided into two groups phylogenetically, phenotypically and ecologically: the group of *Gluconacetobacter liquefaciens* and that of *Gluconacetobacter xylinus*. The genus *Komagataeibacter* was introduced as a new genus originating from the *Gluconacetobacter xylinus* group, with *Komagataeibacter xylinus* as the type species. All fourteen species placed formerly in the group of *Ga. xylinus* have been transferred to this new genus (Yamada, 2014). All four new genera described above possess ubiquinone-10 as their major respiratory quinone (Yamada et al., 2012a; Yukphan et al., 2008, 2009, 2011). Up to date, there are 34 species of *Acetobacter* spp., 16 species of *Gluconobacter* spp., 24 species of *Gluconacetobacter* spp., 14 species of *Komagataeibacter* spp., 8 species of *Asaia* spp., 2 species of *Neokomagataea* spp. and the remaining 8 genera are monotypic, of which only 1 species was isolated (Parte, 2014) so far.

Oxidative fermentation in AAB

Membrane-bound and cytosolic oxidoreductases

Many membrane-bound and cytosolic oxidoreductases were described to be present in AAB (Fig. 1). As known from the completed genome of *Gluconobacter oxydans* ATCC 621H, there are 32 membrane-bound dehydrogenases, 11 with identified and 21 with unidentified substrate specificities (Prust et al., 2005; Richhardt et al., 2013). While membrane-bound enzymes catalyze the oxidations of various alcohols, sugars, sugar alcohols, and sugar acids to provide the primary energy, the cytosolic enzymes seem to play the role in assimilation of the oxidation products in later phase, which is related to the biomass formation (Matsushita et al., 1994; Richhardt et al., 2012; Sakurai et al., 2013). Many membrane-bound enzymes reported from AAB are PQQ- or FAD-dependent proteins containing heme *c* moieties as the electron transfer mediators. PQQ-ADH consists of three subunits: large dehydrogenase subunit containing PQQ and one heme *c*, cytochrome *c* subunit with three hemes *c* and a small subunit of subunit assembly function (Adachi et al., 2007; Masud et al., 2010). PQQ-ADH has been found to possess the binding sites for ubiquinone, which are involved in catalytic function (Matsushita et al., 2008). On the other hand, the nature of the prosthetic group of ALDH, which oxidizes short chain aldehydes, is still unclear. Takemura et al. (1994) reported that the cofactor of ALDH is not PQQ as the PQQ-deficient mutant of *Acetobacter* sp. BPR2001 lost activities of PQQ-dependent enzymes, but ALDH activity remained intact. Thurner et al. (1997) reported heme *b*, [2Fe–2S] cluster and molybdopterin as prosthetic groups of *Komagataeibacter* (*Gluconoacetobacter*) *europaeus* ALDH, while Gómez-Manzo et al. (2010) showed that ALDH from *Ga. diazotrophicus* possessed PQQ, heme *b*, and heme *c* as the prosthetic groups. Further experiments should be conducted to verify the prosthetic groups of ALDH in AAB.

An enzyme catalyzing the oxidation of cyclic alcohols and aliphatic secondary alcohols to their corresponding ketones, PQQ-cyclic alcohol dehydrogenase (PQQ-CADH), has been reported from *Gluconobacter frateurii* CHM9 (Moonmangmee et al., 2001). An important enzyme in the membrane of *Gluconobacter* is

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the PQQ-dependent polyol dehydrogenase known also as PQQ-glycerol dehydrogenase (PQQ-GLDH; Matsushita et al., 2003). PQQ-GLDH has a versatile substrate specificity and has been revealed identical to D-gluconate dehydrogenase (Salusjarvi et al., 2004; Shinagawa et al., 1999), D-arabitol dehydrogenase (Adachi et al., 2001), D-sorbitol dehydrogenase (Hoshino et al., 2003; Miyazaki et al., 2002; Sugisawa and Hoshino, 2002) and GLDH (Ameyama et al., 1985). The general name PQQ-GLDH was given by Matsushita et al. (2003) as the enzyme described as GLDH was the first one isolated (Ameyama et al., 1985), however, it may be referred to as a polyol dehydrogenase in some literature. PQQ-GLDH is capable of catalyzing the oxidation of glycerol, *meso*-erythritol, D-arabitol, ribitol, D-sorbitol, D-mannitol and D-gluconate to produce dihydroxyacetone, L-erytrulose, D-xylulose, L-ribulose, D-fructose and 5KGA, respectively (Adachi et al., 2007; Keliang and Dongzhi, 2006; Matsushita et al., 2003; Moonmangmee et al., 2002a; Toyama et al., 2005).

In D-glucose metabolism, D-glucose is oxidized to glucono-δ-lactone by a membrane-bound PQQ-glucose dehydrogenase (PQQ-GDH), which is then converted to D-gluconic acid (GA) either spontaneously or by a gluconolactonase present in the membrane (Matsushita et al., 1994; Shinagawa et al., 2009). GA is then converted to 2KGA or 5KGA by two different membrane-bound GA-oxidizing enzymes, which perform the competitive reaction in vivo. An FAD-containing gluconate dehydrogenase (FAD-GADH) is responsible for 2KGA formation (Shinagawa et al., 1984; Toyama et al., 2007), while 5KGA is formed by PQQ-GLDH. In some strains of AAB, 2KGA is further converted to 2,5-diketo-D-gluconic acid (25DKGA) by a membrane-bound 2KGA dehydrogenase (Matsushita et al., 1994; Shinagawa et al., 1981). These glucose oxidation products are assimilated by cytoplasmic reductases in the stationary phase of growth (Fig. 1). The resulting products are then subjected to pentose-phosphate pathway as the major route to produce cell mass and the second growth phase is observed. Another membrane-bound enzyme reported in AAB is FAD-dependent D-fructose dehydrogenase, which catalyzes the oxidation of D-fructose to 5-keto-D-fructose (Adachi et al., 2007). The enzyme consists of large dehydrogenase subunit with FAD, the cytochrome *c* subunit, and a small subunit similar to other reported FAD-dependent membrane-bound dehydrogenases (Kawai et al., 2013).

When D-sorbitol is used as substrate, *Gluconobacter* strains will oxidize it to L-sorbose. The enzymes involved in this oxidation have been reported to be both FAD- and PQQ-dependent enzymes. The PQQ-dependent one is the PQQ-GLDH described above, whereas the FAD-dependent D-sorbitol dehydrogenase (FAD-SLDH) is an enzyme specific only to D-sorbitol. This enzyme has 3 subunits similarly to FAD-GADH and FAD-dependent D-fructose dehydrogenase (Toyama et al., 2005). The FAD-SLDH participates in D-sorbitol oxidation only when a high level of D-sorbitol is present, which cannot be completely oxidized by PQQ-GLDH only (Soemphol et al., 2008). There are several membrane-bound and cytosolic oxidoreductases remaining to be identified and characterized in AAB. The recent findings on the newly discovered enzymes from AAB are described in following sections.

Membrane-bound enzymes in "quinate" oxidative fermentation

It has been reported that some *Gluconobacter* strains, such as *G. oxydans* NBRC3244, *Gluconobacter melanogenus* NBRC3292, and *G. oxydans* NBRC3294, possess the membrane-bound quinate dehydrogenase (QDH) which catalyzes the oxidation of quinate to 3-dehydroquinate (DQA). In these strains, 3-dehydroshikimate (DSA), the direct precursor for production of protocatechuic acid (PCA) which is an important antioxidant and anti-inflammatory compound, was the final product of quinate oxidation by the oxidative fermentation. The enzyme responsible for the formation of DSA from DQA has been known as soluble 3-dehydroquinate dehydratase (sDQD) present in cytoplasm (Kleanthous et al., 1992). The accumulation of high amount of DSA observed in the culture medium indicated a possible existence of another type of DQD enzyme, pDQD, which is membrane-associated and different from the sDQD. The activity of pDQD was detected in the membrane fractions at the level of 100-fold higher than the sDQD activity in cytoplasm. Adachi et al. (2008) solubilized and purified pDQD from the membrane fraction of *G. oxydans* NBRC3244 and the enzyme properties were compared with purified sDQD. Both DQDs have similar molecular mass, 191 kDa and 189 kDa for pDQD and sDQD, respectively, however, pDQD is composed of twelve identical subunits of 16 kDa, whereas the sDQD is probably a tetrameric enzyme consisting of four 47 kDa identical subunits. The comparison of N-terminal amino acid sequences of both DQDs with the genomic sequence of *G. oxydans* ATCC 621H (Prust et al., 2005) showed that pDQD matched with the locus *GOX0437*, whereas the sDQD sequence matched with locus *GOX1351*. Both DQDs were similar in enzymatic properties having the optimum pH of 7.5–8.0 with no trace of activity observed at the pH below 5.0 or over 9.0. K_m of pDQD and sDQD for DQA were estimated to be 40 μM and 50 μM, respectively. Both DQDs were considered to be members of type II DQDs and catalyzed the formation of DSA. The membrane-bound pDQD is supposed to co-exist with membrane-bound QDH, oxidizing quinate to DSA via DQA. On the other hand, sDQD is responsible for DSA production which is further subjected to shikimate formation in cytoplasm. The *qdh* gene of 2475 bp was isolated from *G. oxydans* NBRC3244 and heterologously expressed in *Pseudomonas putida* HK5 to confirm its function (Vangnai et al., 2010). Recently, pDQD was successfully overexpressed in *G. oxydans* NBRC3244 (Nishikura-Imamura et al., 2014) reaching more than 100-fold higher level than in the original strain. This high expression level resulted in an increased conversion rate of quinate to DSA in *G. oxydans* NBRC3244 and nearly 100% conversion yield after amending the pH of the medium to 7.3.

G. oxydans NBRC3244 oxidizes quinate to DSA in log phase as described in previous section. DSA accumulated in the medium was found to be converted to PCA in the stationary phase. Although the enzyme responsible for DSA oxidation to PCA in other microorganisms has been known to be the soluble 3-dehydroshikimate dehydratase (sDSD) present in cytoplasm, the rapid conversion observed in *G. oxydans* indicated the existence of a membrane-type DSD (mDSD). Shinagawa et al. (2010) purified the first mDSD ever isolated from membrane fraction of *G. oxydans* NBRC3244 and characterized its catalytic properties. Purification of sDSD was also attempted, but the enzyme seemed to be very labile and was lost during the purification. The purified mDSD is a monomeric enzyme of 76 kDa with no coenzyme requirement and only specific to DSA. No activity was observed with quinate, 3-dehydroquinate, and shikimate.

Oxidative fermentation for pentoses

A new strain of *Ga. liquefaciens* recently isolated from an Argentine water kefir (Adachi et al., 2010) accumulated 25DKGA rapidly when growing in D-glucose-containing medium. 25DKGA was then converted to 4-keto-D-arabonate (4KAB) at stationary phase. Formation of an intermediate, presumably 4-keto-D-arabinose (4KAR), was observed during the oxidation of 25DKGA to 4KAB in the later phase of the growth on glucose-gluconate medium. Two probable sequential enzymatic reactions catalyzing the biotransformation of 25DKGA to 4KAB have been proposed by Adachi et al. (2011b): 25DKGA conversion by a membrane-bound 2,5-diketo-D-gluconate decarboxylase to 4KAR, further metabolized to 4KAB by a membrane-bound 4-keto-D-aldopentose-1-dehydrogenase as shown in Fig. 2. However, the existence of these two membrane-bound enzymes along with the presence of 4KAR is yet to be confirmed.

When D-arabinose and D-arabonate were oxidized by the membrane fraction of *G. suboxydans* NBRC12528, 4KAB was formed as the final product. 4KAR was observed as an intermediate of D-arabinose conversion to 4KAB. This suggested the existence of a D-aldopentose-

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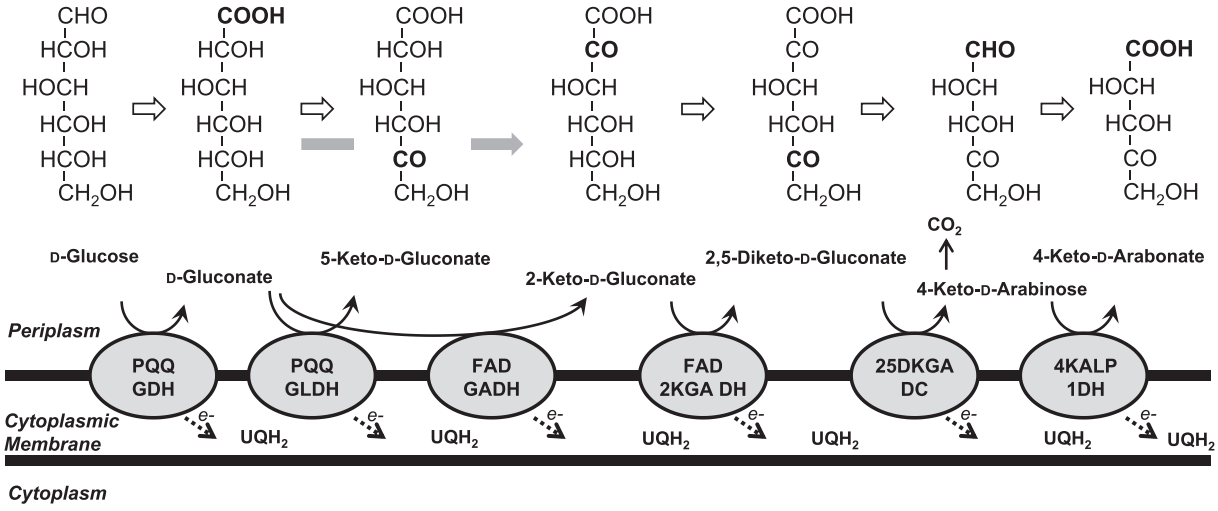

Fig. 2. Proposed D-glucose metabolism in *Gluconobacter*. D-Glucose was found to be oxidized by several membrane-bound enzymes and form 4-keto-D-arabonate as the end product in some *Gluconobacter* strains. PQQ-GDH: PQQ-dependent D-glucose dehydrogenase; PQQ-GLDH: PQQ-dependent glycerol dehydrogenase; FAD-GADH: FAD-dependent D-gluconate dehydrogenase; FAD-2KGA DH: FAD-dependent 2-keto-D-gluconate dehydrogenase; 25DKGA DC: 2,5-diketo-D-gluconate decarboxylase; and 4KALP1DH: 4-keto-D-aldopentose-1-dehydrogenase.

4-dehydrogenase catalyzing the oxidation at C-4 position of D-arabinose. Then 4KAR would be converted to 4KAB by 4-keto-D-aldopentose-1-dehydrogenase (Adachi et al., 2011b). In the case of D-arabonate, the oxidation at C-4 position was suggested to be catalyzed by D-pentonate-4-dehydrogenase (Adachi et al., 2011b). D-aldopentose-4-dehydrogenase recognizes the secondary alcohol group in the D-*erythro*-configuration as it also reacts with D-ribose and D-fructose to form 4-keto-D-ribose (4KRB) and 5-keto-D-fructose, respectively. These novel enzymes have opened the possibility of enzymatic synthesis of new sugar derivatives in sugar chemistry. Enzymatic synthesis of 4KAB has been successfully performed using the membrane fraction of *G. oxydans* NBRC12528 by Adachi et al. (2011a) with three different starting substrates (see Table 1). When D-arabonate is used as substrate, D-pentonate 4-dehydrogenase can oxidize it to 4KAB in a single step reaction. In the case of D-arabinose, D-aldopentose 4-dehydrogenase catalyzes the formation of 4KAR, which is then converted to 4KAB by 4-keto-D-aldopentose 1-dehydrogenase. The formation of 4KAB has been confirmed by IR, ^{1}H NMR, ^{13}C NMR, and a high resolution mass spectroscopy. 4-Keto-D-ribonate (4KRN) was also produced by the same membrane fraction by reacting with D-ribose or D-ribonate. As described above, D-ribose is oxidized by D-aldopentose 4-dehydrogenase, yielding 4KRB, and then further oxidized by 4-keto-D-aldopentose 1-dehydrogenase resulting in the formation of 4KRN as the final product from membrane fraction reaction. It was found that the enzyme systems also oxidized 2′-deoxy-D-ribose to form 2′-deoxy-4KRN (Adachi et al., 2013). When D-ribonate is a substrate, D-pentonate 4-dehydrogenase oxidizes it to 4KRN as the final product (see Table 1). 4KRN produced from membrane-bound enzyme reaction was identified by using IR, H–H COSY, and high resolution mass spectroscopies. As described in this section, there are still at least four membrane-bound enzymes to be purified and characterized from *Gluconobacter*.

Thermotolerant AAB

AAB are mesophilic bacteria which have the optimum temperature for normal growth at around 30 °C. The growth is dramatically decreased at higher temperatures and there is usually no growth at the temperature over 34 °C. The thermotolerant strains, however, will continue to grow at 37 °C and some strains can even grow at temperatures up to 42 °C (Soemphol et al., 2011). During the fermentation, heat is generated as a part of biological fermentation activity and the cooling system is required to maintain the optimum temperature for bacterial growth and production. The mesophilic character of AAB, which requires a strict temperature control, poses considerable disadvantage for industrial applications. Many AAB isolated in tropical countries possess the thermotolerant ability, which makes them more suitable for industrial applications. Thermotolerant strains that have been introduced for production of many valuable products in the past two decades are summarized in Table 2.

Thermotolerant strains for vinegar production

Thermotolerant AAB have been isolated from several sources and their properties were studied intensively for over a decade. Saeki et al. (1997) isolated thermotolerant AAB, which were classified as *Acetobacter rancens* subsp. *pasteurianus*, *Acetobacter lovaniensis* subsp.

Table 1
Enzymatic reactions of some newly discovered enzymes related to the oxidative fermentation from AAB.

Substrate	Enzyme	Product	References
3-Dehydroquinate	3-Dehydroquinate dehydratase	3-Dehydroshikimate	Adachi et al. (2008)
3-Dehydroshikimate	3-Dehydroshikimate dehydratase	Protocatechuate	Shinagawa et al. (2010)
D-ribose	D-aldopentose 4-dehydrogenase	4KRB	Adachi et al. (2011a,b)
2′-Deoxy-D-ribose	D-aldopentose 4-dehydrogenase	2′-Deoxy-4KRB	Adachi et al. (2013)
D-fructose	D-aldopentose 4-dehydrogenase	5-Keto-D-fructose	Adachi et al. (2011a,b)
D-arabonate	D-pentonate 4-dehydrogenase	4KAB	Adachi et al. (2011a,b)
4KRB	4-Keto-D-aldopentose 1-dehydrogenase	4KRN	Adachi et al. (2011a,b)
2′-Deoxy-4KRB	4-Keto-D-aldopentose 1-dehydrogenase	2′-Deoxy-4KRN	Adachi et al. (2013)
4KAR	4-Keto-D-aldopentose 1-dehydrogenase	4KAB	Adachi et al. (2011a,b)

Please cite this article as: Saichana N, et al, Acetic acid bacteria: A group of bacteria with versatile biotechnological applications, Biotechnol Adv (2014), http://dx.doi.org/10.1016/j.biotechadv.2014.12.001

Table 2
Thermotolerant acetic acid bacteria and their applications.

Strains	Growth and Production[a]	Product (s)	References
G. frateurii CHM16	37 °C	D-fructose	Moonmangmee et al. (2000)
G. frateurii CHM54	37 °C	L-sorbose	Moonmangmee et al. (2000)
G. frateurii CHM43	37 °C	L-erythrulose	Moonmangmee et al. (2002a)
G. frateurii THD32	37 °C	L-sorbose	Toyama et al. (2005)
G. frateurii CHM43Ad	38.5–40 °C	L-sorbose	Hattori et al. (2012)
Gluconobacter sp. THE42	37 °C	2KGA, 5KGA	Saichana et al. (2009)
Gluconobacter sp. THF55	37 °C	2KGA, 5KGA	Saichana et al. (2009)
Gluconobacter sp. THG42	37 °C	2KGA, 5KGA	Saichana et al. (2009)
A. tropicalis SKU1100	37 °C	CPS, EPS	Deeraksa et al. (2005); Moonmangmee et al. (2002b)
Acetobacter sp. I14-2	37 °C	Acetic acid	Lu et al. (1999)
A. pasteurianus SKU1108	37 °C	Acetic acid	Saeki et al. (1997)
A. pasteurianus MSU10	37 °C	Acetic acid	Kanchanarach et al. (2010a)
A. tropicalis CWBI-B418	38 °C	Acetic acid	Ndoye et al. (2006)
A. pasteurianus CWBI-B419	38 °C	Acetic acid	Ndoye et al. (2006)
A. pasteurianus TI[b]	39–40 °C	Acetic acid	Matsutani et al. (2013)
A. pasteurianus TH-3[b]	39–40 °C	Acetic acid	Matsutani et al. (2013)
A. pasteurianus SL13E-2	37 °C	Acetic acid, CPS	Perumpuli et al. (2014a, 2014b)
A. pasteurianus SL13E-3	37 °C	Acetic acid, CPS	Perumpuli et al. (2014a, 2014b)
A. pasteurianus SL13E-4	37 °C	Acetic acid, CPS	Perumpuli et al. (2014a, 2014b)

[a] Temperatures for optimum growth and production.
[b] Thermoadapted strain of *A. pasteurianus* SKU1108.

lovaniensis, *Acetobacter aceti* subsp. *liquefaciens*, and *Acetobacter xylinum* subsp. *xylinum*. These strains produced acetic acid at 38 to 40 °C. They also oxidized high concentrations of ethanol (up to 9%), at a high fermentation rate without any noticeable lag time. Acetic acid fermentation at higher temperatures was successful in both submerged culture and static culture, which is advantageous over the mesophilic strains. The *A. lovaniensis* subsp. *lovaniensis* was later reclassified as *A. pasteurianus* SKU1108, which is one of the best thermotolerant vinegar producers (Kanchanarach et al., 2010a, 2010b). Lu et al. (1999) reported a thermotolerant acetic acid bacterium, *Acetobacter* sp. I14-2, with high ability to produce acetic acid at high temperature. This I14-2 strain possesses remarkable thermotolerance with yields of 85 and 82% acetic acid production when it is cultured for 6 days at 35 and 37 °C, respectively. The yield at 37 °C achieved with *Acetobacter* sp. I14-2 is comparable with that of *A. pasteurianus* SKU1108 reported earlier (Saeki et al., 1997). Ndoye et al. (2006) isolated two outstanding thermotolerant AAB from fruits in Sub-Saharan Africa. These new isolates were designated as *Acetobacter tropicalis* CWBI-B418 and *A. pasteurianus* CWBIB419 according to their 16S rDNA sequences. Both strains grew very well at 35 °C and showed the normal growth at 40 and 45 °C, although the growth in a medium containing 2.5% ethanol and 0.5% acetic acid was slightly lower at 38 °C. CWBI-B419 was the most outstanding strain as it showed highest level of acetate production at 38 °C. The strains CWBI-B418 and CWBI-B419 also showed high acetic acid resistance at this temperature. It was found that the enzymes responsible for acetic acid production in these strains, ADH and ALDH, are more thermostable than those from mesophilic strains (Lu et al., 1999). The thermotolerant nature of the enzymes accommodated in thermotolerant strains was also found by Kanchanarach et al. (2010b) and Perumpuli et al. (2014a) who showed that ADHs from thermotolerant strains were more stable at higher temperature than ADHs from mesophilic strains. It was also found that in thermotolerant strains, the formation of pellicles, capsular polysaccharides covering the cells helping them to float on the medium surface, also played a part in thermotolerance (Deeraksa et al., 2005; Kanchanarach et al., 2010a). Recently, new thermotolerant strains assigned as *A. pasteurianus* SL13E-2, SL13E-3, and SL13E-4 and *G. frateurii* SL13-5, SL13-6, SL13-7, and SL13-8 were isolated from coconut water vinegar from Sri Lanka (Perumpuli et al., 2014a). The strains SL13E-2, SL13E-3, and SL13E-4 could produce 2.5% acetic acid from 4% ethanol at 40 °C and 4% acetic acid from 6% ethanol at 37 °C, which is comparable to *A. pasteurianus* SKU1108, a well-known thermotolerant acetic acid producer strain. The three strains showed higher ethanol tolerance than *A. pasteurianus* SKU1108 as they produced more acetic acid at 37 °C; however, the strain SKU1108 still showed the acid production at 40 °C with 6% ethanol, whereas all three strains did not. ADHs in all strains were found to be thermotolerant up to 55 °C, at which they were comparable to ADH of the strain SKU1108. These thermotolerant strains were used for coconut water vinegar production at 37–40 °C and it was found that the strains SL13E-2 and SL13E-3 are equally good strains for the production as well as SKU1108 strain, however, SL13E-4 showed a lower productivity.

Thermotolerant Gluconobacter strains for polyol oxidative fermentation

Among other AAB, *Gluconobacter* is a genus of AAB, which has outstanding oxidative fermentation ability for sugars, sugar alcohols, and sugar acids (Gupta et al., 2001; Mamlouk and Gullo, 2013; Moonmangmee et al., 2000). Production of D-fructose from D-mannitol in *Gluconobacter* was known to be catalyzed by a general polyol dehydrogenase, so-called PQQ-GLDH (Adachi et al., 2003a, 2003b; Matsushita et al., 2003; Moonmangmee et al., 2000). *G. frateurii* CHM16 has been reported to have an ability to produce D-fructose at 37 °C, at which none of mesophilic strains could grow nor produce any product. D-Mannitol was almost completely oxidized within 24 h. The strain showed poor growth at 38 °C and could not grow at 39 °C. The yields of D-fructose formation after 24 h of fermentation were 100% and 80% at 30 °C and 37 °C, respectively (Moonmangmee et al., 2000). Resting cells reaction of *G. frateurii* CHM16 showed the highest amount of D-fructose at 37 °C whereas the meshophilic strain accumulated highest amount of D-fructose at 30 °C.

G. frateurii CHM43 has been reported to produce high amount of L-erythrulose from *meso*-erythritol (Moonmangmee et al., 2002a). This strain produced more L-erythrulose at 37 °C than at 30 °C, which was considered as the superior characteristics for industrial applications, as the temperature in a large fermentor often raises over 30 °C in regular fermentation without cooling. These conditions would severely affect the production when using mesophilic strains. L-Erythrulose was accumulated to 90% within 24 h at 30 °C, but 34% of the product was consumed after 48 h of cultivation at this temperature. This consumption was not observed when the cells were grown at 37 °C, where 100% conversion was observed

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within 48 h. The conversion of *meso*-erythritol to L-erythrulose is catalyzed by PQQ-GLDH, which forms the product according to Bertrand–Hudson's rule (Adachi et al., 2003b).

It has been previously reported that a thermotolerant *G. frateurii* CHM54 produced significantly high amount of L-sorbose from D-sorbitol (Moonmangmee et al., 2000). As described in Membrane-bound and cytosolic oxidoreductases, L-sorbose production in *Gluconobacter* is catalyzed by two enzymes, FAD-SLDH (Shinagawa et al., 1982; Soemphol et al., 2008; Toyama et al., 2005) and PQQ-GLDH (Ameyama et al., 1985; Matsushita et al., 2003). Thermotolerant *G. frateurii* THD32 able to produce L-sorbose at 37 °C by FAD-GADH has been isolated by Toyama et al. (2005). Hattori et al. (2012) reported that among 8 thermotolerant *G. frateurii* CHM strains previously isolated by Moonmangmee et al. (2000), CHM43 strain showed the best growth on D-sorbitol medium at 37 °C and could grow even at 39 °C, where the other strains could not grow at all. Although it was reported that the CHM54 strain produced more L-sorbose than the other CHM strains at 37 °C, the dominant growth at 39 °C of strain CHM43 was considered a superior characteristic amenable to further improvement. The CHM43 strain adapted to higher temperature was obtained by repeated cultivation at 38.5 °C for several generations. The adapted strain showed higher growth and L-sorbose production than the original CHM43 strain at temperature between 38.5–40 °C, at which typical thermotolerant strains of *Gluconobacter* show poor growth. It was found that the activity of PQQ-GLDH of both wild-type and adapted strains was decreased when the temperature increased, but GLDH activity was recovered by addition of PQQ (Hattori et al., 2012). PQQ production in the adapted strain was higher than that in the original CHM43 strain suggesting that the adapted strain could keep GLDH in holo-form by the increased PQQ production resulting from adaptive mutations.

Thermotolerant strains for 2-keto-D-gluconic acid and 5-keto-D-gluconic acid production

Gluconobacter strains produce D-gluconic acid (GA) from D-glucose, which is accompanied by the pH drop of the medium preventing growth of other competitive bacteria. GA is further converted to 2KGA or 5KGA that are both assimilated later in the stationary phase. This property is an important characteristic as the ketogluconic acid is not be consumed during the production process. The production ratio of 2KGA and 5KGA depends on individual strain and growth conditions (Ano et al., 2011; Shinagawa et al., 1983) as the production relies on the particular ratio of FAD-GADH and PQQ-GLDH activities (see Membrane-bound and cytosolic oxidoreductases). 2KGA is applicable as a building block in the chemical synthesis of heterocyclic compounds and for various regioselective and stereoselective chemical reactions (Stottmeister et al., 2005). 2KGA was found in some species of *Gluconobacter*, *Pseudomonas*, *Serratia* and *Klebsiella* (Matsushita et al., 1982). 2KGA is a dominant product in most *Gluconobacter* spp., including thermotolerant strains isolated from Thailand (Moonmangmee et al., 2000), due to a higher affinity of FAD-GADH to D-gluconate compared to PQQ-GLDH. *Gluconobacter dioxyacetonicus* NBRC3271 has been reported as a mesophilic 2KGA-producing strain, which possibly possesses two types of FAD-GADH (Toyama et al., 2007). It was suggested that exclusive 2KGA production could be achieved by deletion of the PQQ-GLDH genes.

5KGA is a useful raw material applicable for the production of tartaric acid, xylaric acid, or 4-hydroxy-5-methyl-dehydrofuranone-3, valuable flavor compound used in food industry (Salusjarvi et al., 2004). Moreover, 5KGA is applicable for vitamin C production by the Gray's method (Gray, 1945a, 1945b), which is considered an environmental-friendly process because it requires milder condition for the operation than the Reichstein's method (Reichstein and Grüssner, 1934), currently used in vitamin C production industry. 5KGA production has been exclusively found in *Gluconobacter* (Toyama et al., 2007), although *Xanthomonas campestris* was reported to possess a secondary-alcohol dehydrogenase, which oxidized GA to 5KGA when heterologously expressed in *E. coli*. However, *X. campestris* did not produce any detectable 5KGA naturally and it was suggested that the corresponding gene was not expressed in this bacterium (Salusjarvi et al., 2004). *G. suboxydans* NBRC12528 is a strain with dominant 5KGA production; however its mesophilic character is a major drawback for industrial applications as it only shows good production at the temperature below 30 °C (Shinagawa et al., 1999). Saichana et al. (2009) performed the screening of thermotolerant strains, formerly isolated in Thailand by Moonmangmee et al. (2000), for the high ability to produce 5KGA. The strains THE42, THF55, and THG42 showed 5KGA production at 37 °C, at which the mesophilic 5KGA producer *G. suboxydans* NBRC12528 could not grow at all. Although 2KGA was still a dominant product produced by these strains, disruption of FAD-GADH diminished 2KGA production and the strains exclusively produced 5KGA. The growth and 5KGA production in these strains decreased at 37 °C along with the loss of holo-PQQ-GLDH in the membrane fractions. Addition of Ca^{2+} was found to restore the holo-PQQ-GLDH and resulted in higher production of 5KGA at 37 °C.

Polysaccharide production by thermotolerant AAB

The most well-known polysaccharide produced by AAB is cellulose. *Komagataeibacter xylinus* (formerly known as *Ga. xylinus* or *A. xylinum*) is a major producer of bacterial cellulose (Embuscado et al., 1994a, 1994b). Cellulose production has been studied in detail for decades and developments on the production are currently focusing on the improvement of its properties, selection of new carbon sources to reduce the production cost and isolation of new strains (Carreira et al., 2011; Castro et al., 2011; Chen et al., 2013; Gomes et al., 2013; Lin et al., 2013; Ruka et al., 2012; Trovatti et al., 2011; Zeng et al., 2011). Some strains of AAB, such as *G. frateurii* TMW 2.767, *G. cerinus* DSM 9533 T, *Neoasaia chiangmaiensis* NBRC 101099, and *Kozakia baliensis* DSM 14400, can produce high amounts of fructans, polymers of D-fructose applicable in food industries (Jakob et al., 2013). A novel pellicle polysaccharide from thermotolerant *A. tropicalis* SKU1100 was reported to be composed of glucose, galactose, and rhamnose in the molar ratio of 1:1:1 (Deeraksa et al., 2005; Moonmangmee et al., 2002b). Production of the pellicles (capsular polysaccharides, CPS) was observed at higher temperatures up to 40 °C in static culture, though the production decreased in shaking cultures. The purified polysaccharide showed the average molecular mass of 120 kDa. The genes for polysaccharide formation were identified as *polABCDE*. Slippage mutations were found at a region of *polE* gene, where repeating C nucleotides were observed. This mutation resulted in a release of polysaccharide to the medium as extracellular polysaccharide (EPS) and the strain lost its pellicle formation. Such a phenotype was also observed in the Δ*polE* mutant. The Δ*galE* mutant lacking D-galactose formation also produced EPS, but with a much larger molecular mass higher than 400 kDa (Deeraksa et al., 2006). The structures of wild-type CPS, Δ*polE* mutant EPS, and Δ*galE* mutant EPS were reported by Ali et al. (2011). The structures of wild-type CPS and Δ*polE* mutant EPS were similar as they had a branched hexasaccharide repeating unit composed of 2 mol of 2,3-α-L-rhamnopyranosyl, and 1 mol each of 6-β-D-galactopyranosyl and 2-α-D glucopyranosyl residues. They branched at the rhamnosyl residues, with terminal-β-D-galactofuranosyl and terminal-α-D-glucopyranosyl residues. The EPS of Δ*galE* mutant showed a branched tetrasaccharide repeating unit composed of 2,3-α-L-rhamnopyranosyl, 2-α-glucopyranosyl, and 3-α-L-rhamnopyranosyl residues and a terminal-α-D-glucopyranosyl residue. It was proposed that PolE may control the switching of EPS to CPS by adding some residues, such as β-D-galactopyranosyl residue, to 2,3-α-L-rhamnopyranosyl residue to make 2,3,4-α-L-rhamnosyl residue found in CPS. The newly isolated thermotolerant *A. pasteurianus* SL13E-2, SL13E-3, and SL13E-4 from Sri

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Lanka showed the production of pellicles as well as acetic acid production (Perumpuli et al., 2014b). Pellicles of strain SL13E2 were composed of rhamnose and glucose at the ratio of 1:8, whereas the strain SL13E-4 produced pellicles consisting of rhamnose, glucose, and xylose at ratio of 1:5:2. The strains SL13E-3 and SKU1108 were found to produce pellicles consisting of rhamnose, glucose, and galactose at the ratio of 2:2:1 and 1:5:2.5, respectively. Pellicle production in all newly isolated strains was inducible by ethanol, however, this induction was not observed in SKU1108. These new polysaccharides have potential to serve as the new materials in many applications in food, cosmetic, and medical industries and they should be studied in details to be used for further purposes.

Genetic adaptation and engineering approach for strain improvement

Genetic adaptation of thermotolerant AAB

It has been known that some AAB possess high mutability during growth at certain conditions due to temporal acclimation or heritable adaptation (Azuma et al., 2009; Deeraksa et al., 2005). This genetic instability causes the loss of various useful phenotypic characteristics of AAB, such as ethanol oxidation, acetate resistance, pellicle formation and cellulose biosynthesis (Matsutani et al., 2013). Azuma et al. (2009) have shown that *A. pasteurianus* NBRC3283 could be adapted to become a thermotolerant bacterium by repeated cultivation at unviable high temperature and obtained a thermotolerant strain NBRC3283-01, which was able to grow at 42 °C. This thermotolerant strain exhibited a stable thermotolerant character after several passages at 30 °C, suggesting that the adapted strain had acquired mutations in the genome in order to survive at higher temperatures. Hattori et al. (2012) have performed an adaptive mutagenesis of *G. frateurii* CHM43, an outstanding thermotolerant strain for L-sorbose fermentation, and obtained the thermoadapted strain called CHM43AD as described in Thermotolerant *Gluconobacter* strains for polyol oxidative fermentation. Wild type *A. pasteurianus* SKU1108 has been known to produce high amount of acetic acid at high temperatures up to 38 °C, but lower rate of acetic acid production was observed when the cultivation temperature rose to 39 °C. By repeated cultivation at 39 °C for 1150 h (11 passages to new medium), the TI strain was obtained. Moreover, an adapted strain TH-3 was obtained by stepwise adaptation to higher temperature by repeated cultivation from 38.5 to 40 °C. TI and TH-3 strains exhibited the improved growth characteristics as they grew normally at 39 °C and 40 °C both under fermenting (with ethanol) and non-fermenting (without ethanol) conditions. Whole genome mapping of the wild-type SKU1108 and the two adapted strains was performed and intensive comparison of the genomes was carried out by Matsutani et al. (2013). Mutations at 6 and 11 positions on the genomes of TI and TH-3, respectively, were identified. Three genes, amino acid transporter (APT_1698), transcriptional regulator MarR (APT_2081) and C4-dicarboxylate transporter (APT_2237) were mutated in both TI and TH-3 strains. Disruption of *marR* and *APT1698* was performed to observe the phenotypes of the mutants comparing to the wild-type and two adapted strains. The *marR* disruptant showed intermediate growth characteristics of the wild-type and TI strain and exhibited much higher acetate resistance than the wild-type. Disruption of *marR* also induced expression of some *marR*-regulated genes, which are supposed to be responsible for increased ethanol oxidation or acetate resistance, but not involved in thermotolerance. Disruption of *APT1698* have pointed to involvement of amino acid transporter in thermotolerance as the mutant could produce acetic acid at 39 °C, the temperature at which the wild-type could not grow well. This mutant strain also showed a delayed growth, indicating that *APT1698* was also related to the maintenance of prolonged acetic acid resistance phase. It was suggested that the effects of single gene disruption in the *marR* and *APT1698* genes were involved in ethanol oxidation and/or acetate-resistance ability, and the thermotolerance and/or the maintenance of a prolonged acetic acid-resistance phase, respectively. However, these effects of single gene disruption on the thermotolerance and/or fermentation ability were smaller than on those of TI and TH3 strains, which probably had the combined effect of multi-gene disruption. It has been reported that many genes involved in thermotolerance of *A. tropicalis* SKU1100 also played a part in acetic acid resistance (Soemphol et al., 2011). Some genes not directly involved in thermotolerance, such as genes participating in cell wall or cell membrane biosynthesis, also affect the growth properties under acetic acid stress or higher temperatures. Qi et al (2014) constructed an adapted mutant of *A. pasteurianus* CICIM B7003 by UV mutagenesis under acid stress condition. The mutant obtained, called B7003-02 strain, exhibited an ability to accumulate 103.81 ± 1.17 g L^{-1} of acetic acid within 160-h of a batch cultivation, which was 49.2% higher than that of the wild type. Repeated batch fermentations in a Frings 8-L acetator were carried out and high-acidity vinegars with 90 ± 0.39 g L^{-1}acetic acid were produced. Genetic adaptation of AAB is thus a promising way to develop better strains serving for industrial applications.

Transposon mutagenesis in AAB

Transposable elements, such as Tn*5* or Tn*10*, have been used as tools for mutation studies and locating the genes of interest. In AAB, tools for transposon mutagenesis have been developed and applied in many studies for a decade. Tn*5* has been used for mutation studies in *Escherichia coli*, *Salmonella typhimurium*, *Caulobacter crescentus*, *Erwinia herbicola*, *Vibrio fischeri*, *Rhizobium meliloti*, *Klebsiella pneumonia*, *Agrobacterium* species, and *Azotobacter* species (Gupta et al., 2006). Simon et al. (1983) developed a new vector, pSUP2021, carrying Tn*5* for the insertion of foreign genes into the genomes of gram-negative bacteria not closely related to *E. coli*. This system required an *E. coli* donor strain S17-1 having the transfer genes of the broad host range IncP-type plasmid RP4 integrated in its chromosome. The system could be applied into any gram-negative bacterium as a recipient for DNA transfer by conjugation. The mobilizable pSUP2021 was derived from the commonly used *E. coli* vectors pACYC184, pACYC177, and pBR325, and contained kanamycin resistance gene cassette as a marker. Plasmid pSUP2021 is unable to replicate in strains outside the enteric bacterial group, which ensures that the transposon randomly integrates into the recipient chromosome. Deeraksa et al. (2005) performed a Tn*10* mutagenesis to locate the pellicle polysaccharide biosynthesis genes in *A. tropicalis* SKU1100. The mobilizable plasmid pSUP2021Tn*10* was constructed by inserting Tn*10* from the transposon vehicle phage λNK1323 into pSUP2021. Conjugation was carried out using *E. coli* S17-1 bearing pSUP2021Tn*10* as a donor strain and *A. tropicalis* R-strain as a recipient strain. The transconjugants were selected on agar plates with tetracycline as a selectable marker and nalidixic acid as a suppresser of *E. coli* growth. The mutant deficient in pellicle formation was selected and the location of the Tn*10* insertion site was identified using in vitro cloning strategy. Soemphol et al. (2011) identified 24 genes involved in thermotolerance in *A. tropicalis* SKU1100 by using Tn*10* mutagenesis. The Tn*10* inserted genes were identified by Thermal Asymmetric Interlaced — PCR technique. Gupta et al. (2006) induced mutation on *G. oxydans* ATCC 9937 and obtained a mutant deficient in glucose dehydrogenase activity by Tn*5* mutagenesis. Transfer of Tn*5* to *G. oxydans* ATCC 9937 cells was carried out by conjugation method with *E. coli* S17-1 bearing pSUP2021 as the donor strain. The transconjugants were selected on the selective medium containing kanamycin and nalidixic acid. As mentioned above, transposon mutagenesis can be applied for mutation studies in AAB by conjugative DNA transfer very effectively.

Gene expression systems in AAB

Good expression system is a crucial factor for metabolic engineering involving enhancement of specific gene expression in microorganisms. For AAB, homologous and heterologous expressions of genes have

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been studied in several strains. The discoveries of many indigenous plasmids in AAB and available shuttle vectors for AAB have been summarized by Gupta et al. (2001). Fukaya et al. (1985) have reported the distribution of indigenous plasmids in 27 strains of *Acetobacter* and 23 strains of *Gluconobacter*. Creaven et al. (1994) developed the conditions for effective transformation of *Gluconobacter oxydans* subsp. *suboxydans* by electroporation method. This finding has facilitated gene transfer to *Gluconobacter* and also other AAB as electroporation is a very simple method for both circular or linear form of DNA to be introduced. Expression systems, which require suitable shuttle vector and efficient promoter, have also been developed for AAB in the past decades. Tonouchi et al. (1994) constructed a shuttle vector pSA19 by connecting pAH4, a plasmid isolated from a cellulose-producing *Acetobacter* sp. BPR2001, with pUC18. The plasmid pSA19 carrying ampicillin resistance gene is very stable in the strain BPR2001 and is suitable for gene expression and gene studies in this strain as well as other related strains. Expression of the protein of interest by pSA19 is controlled by the *lac* promoter from pUC18, which was found to be effective in AAB. Saito et al. (1997) constructed a new shuttle vector pFG15A by joining pF4 isolated from *G. oxydans* T-100 to a DNA fragment containing *E. coli ori* and a kanamycin resistance cassette from a commercial pUC19 based cloning vector pHGS298. This vector has been used in co-expression of L-sorbose dehydrogenase and L-sorbosone dehydrogenase to produce 2-keto-L-gulonate from D-sorbitol in *G. oxydans* G624 under the control of their native promoters. The expression was found to be enhanced by replacement of the native promoter with *E. coli* P_{tufB} and P_{tac} or P_L of bacteriophage Lambda. Trcek et al. (2000) constructed a shuttle vector pJT2 by combining pJK2-1 isolated from *A. europaeus* JK2 and pUC18 and successfully used it for transformation in this strain. Tonouchi et al. (2003) developed new expression plasmids pSG8 for use in *Gluconobacter* by ligating pAG5, a cryptic plasmid from *G. oxydans* NBRC3171, to pUC18 at *Hin*dIII site. The pSG8 was then digested with *Eco*RI to remove an unnecessary region of 2 kb and self-ligated to create pSG6. The plasmids were found to be stable with the copy number of 10 per genome with the same transformation efficiency as pSA19 formerly created from an *Acetobacter* cryptic plasmid. The broad host range plasmid pBBR1MCS was constructed by Kovach et al. (1994). This vector contains chloramphenicol resistance gene as a selectable marker and *lac* promoter for gene expression. The plasmid was stable with a versatile host range in gram-negative bacterial group. However, the chloramphenicol resistance feature has limited application due to natural resistance of many gram-negative bacteria to this antibiotic. The derivatives of pBBR1MCS were therefore constructed to carry different antibiotic resistance genes as selectable markers (Kovach et al., 1995). Four plasmids, pBBR1MCS2, pBBR1MCS3, pBBR1MCS4, and pBBR1MCS5, were constructed with kanamycin, tetracycline, ampicillin, and gentamycin resistance genes, respectively. These plasmids are also compatible with DNA transfer by conjugation, which is a useful feature for some AAB strains in which DNA transfer by electroporation was not effective. The plasmids were found to be able to replicate in the following microorganisms: *A. xylinum*; *Alcaligenes eutrophus*; *Bartonella bacilliformis*; *Bordetella* spp.; *Brucella* spp.; *C. crescentus*; *E. coli*; *Paracoccous denitrificans*; *Pseudomonas fluorescens*; *P. putida*; *R. meliloti*; *Rhizobium leguminosarum* bv. *viciae*; *Rhodobacter spehaeroides*; *Salmolella typhimurium*; *Vibrio cholera*; *X. campestris*. Marx and Lidstrom (2001) constructed several versatile broad-host-range vectors for cloning and expression in methylotrophs and other gram-negative bacteria. Plasmid pCM62 and pCM66, which carry tetracycline and kanamycin resistance genes, respectively, were constructed by ligating a transferable and selectable region of a small IncP plasmid pTJS75 with a polylinker and ColE1 *ori* region of pUC19 (Marx and Lidstrom, 2001; Schmidhauser and Helinski, 1985). The vectors pCM62 and pCM66 have a high copy number in *E. coli* with small size of about 7 and 8 kb, respectively. These plasmids were also selectable by blue–white screening with *lacZα* complementation with conjugative mobilization between bacterial species. Both plasmids were found to be stable in many α-, β-, and γ-proteobacteria, such as *Agrobacterium tumefaciens*, *Methylobacterium* strains CM4 and DM4, *Rhodobacter sphaeroides*, *Ralstonia eutropha*, *Methylococcus capsulates*, and *Pseudomonas aeruginosa*. It was found that pCM62 can replicate in *Acetobacter* and was used for expression of genes in *A. tropicalis* SKU1100 and *A. pasteurianus* SKU1108 effectively (Deeraksa et al., 2005; Soemphol et al., 2011; Tasanapak et al., 2013). Merfort et al. (2006) enhanced the promoter efficiency of pBBR1MCS5 by introducing the promoter of elongation factor TU (P_{tufB}) and the GDH promoter (P_{gdh}) into pBBR1MCS5 for PQQ-GLDH expression. The expression efficiencies of the two new promoter constructs were found to be increased by both promoters and the production of 5KGA, which is the product of PQQ-GLDH reaction, was increased by 39% and 24% from P_{gdh} and P_{tufB}, respectively. Schleyer et al. (2008, 2009) have further developed a series of pBBR1MCS5 derivatives containing the strong promoter P_{tufB} to facilitate gene expression in *Gluconobacter*. The new plasmids were designated as pEXGOX-G, pEXGOX-A, and pEXGOX-K, which have gentamycin, ampicillin, and kanamycin resistance genes as the selectable markers, respectively. The advantages of the newly constructed plasmids are favorable for further applications as they have small sizes with a variety of unique restriction sites, and contain *Swa*I site, which will provide the blunt-ended vector after cleavage by *Swa*I for ligation with blunt-ended PCR product, of which the fragment started with the start codon ATG. Zhang et al. (2010) constructed a shuttle vector, pZL1 from a cryptic plasmid pGOX3 found in *G. oxydans* DSM2003 and *E. coli* cloning vector pUC18. The plasmid was found to be very stable even in the absence of antibiotic selective pressure. The copy number of pZL1 in *E. coli* was much higher than for pBBR1MCS5. The plasmid was successfully used for expression in *G. oxydans* DSM2003 under the control of the *tufB* promoter. Kallnik et al. (2010) developed two new derivatives of pBBR1MCS2 by introducing the promoter regions of *gox0264* and *gox0452*, the genes encoding ribosomal proteins L35 and L13, respectively. The resulting plasmids, namely pBBR1p264 and pBBR1p452, were tested for the promoter activities. It was found that the promoter of *gox0264* was a strong promoter and the *gox0452* was a moderate promoter, whereas the *lac* promoter of original pBBR1MCS2 was classified as a weak promoter compared to the new promoters introduced. Obranić et al. (2013) improved the broad host range plasmid pBBR1MCS derivatives by introducing an *Nde*I site to the start codon of *lacZα* gene on the vectors in order to introduce the DNA fragment for expression directly to the position adjacent to the *lac* promoter on the plasmids. The fragment to be expressed by these vectors should have *Nde*I site introduced to the start codon. The resulting plasmids were designated as pBBR1MCS2_START, pBBR1MCS3_START, pBBR1MCS4_START, and pBBR1MCS5_START, respectively. Shi et al. (2014) carried out a screening experiment to find effective promoter from *G. oxydans* DSM2003. The promoter region of *gox0169* encoding a hypothetical protein was isolated as a best promoter from 710 promoter clones in the screening. The expression plasmid, pBBR1*pgHp0169* was then constructed as a broad host range vector for *Gluconobacter*. The $P_{gox0169}$ was found to be a stronger promoter than the P_{tufB} and it was subsequently used as a promoter for expression of FAD-GADH genes in *G. oxydans* DSM2003. The production of 2KGA by the overexpressed FAD-GADH under the control of $P_{gox0169}$ was found to reach the yield of 98.3%. Plasmids constructed for protein expression in AAB are summarized in Table 3.

Conclusion

Many applications of AAB have been reported and developed over the past decades. The most important characteristic is still the ability to produce acetic acid from ethanol. While acetic acid production in European countries is conducted by mesophilic AAB, thermotolerant strains exhibiting the ability to grow at higher temperatures are preferable for production in tropical countries as well as in Africa, in order to

Please cite this article as: Saichana N, et al, Acetic acid bacteria: A group of bacteria with versatile biotechnological applications, Biotechnol Adv (2014), http://dx.doi.org/10.1016/j.biotechadv.2014.12.001

Table 3
Plasmid vectors for expression in acetic acid bacteria.

Plasmids	Relevant trait (s)	Replicable	References
pSA19	Am^r, P_{lac}	*Acetobacter* sp. BPR2001	Tonouchi et al. (1994)
pGF15A	Km^r, P_{tufB}, P_{tac}, P_L	*G. oxydans* G264	Saito et al. (1997)
pJT2	Am^r, P_{lac}	*A. europaeus* JK2	Trcek et al. (2000)
pSG8, pSG6	Am^r, P_{lac}	*Gluconobacter* spp.	Tonouchi et al. (2003)
pBBR1MCS	Cm^r, P_{lac}, *mob*	Broad host range	Kovach et al. (1994)
pBBR1MCS2	Km^r, P_{lac}, *mob*	Broad host range	Kovach et al. (1995)
pBBR1MCS3	Tc^r, P_{lac}, *mob*	Broad host range	Kovach et al. (1995)
pBBR1MCS4	Am^r, P_{lac}, *mob*	Broad host range	Kovach et al. (1995)
pBBR1MCS5	Gm^r, P_{lac}, *mob*	Broad host range	Kovach et al. (1995)
pCM62	Tc^r, P_{lac}, *oriT*, *traJ′*	Broad host range of proteobacteria	Marx and Lidstrom (2001)
pCM66	Km^r, P_{lac}, *oriT*, *traJ′*	Broad host range of proteobacteria	Marx and Lidstrom (2001)
pBBR1MCS5P_{tufB}	Gm^r, P_{tufB}, *mob*	Broad host range	Merfort et al. (2006)
pBBR1MCS5P_{gdh}	Gm^r, P_{gdh}, *mob*	Broad host range	Merfort et al. (2006)
pEXGOX-G	Gm^r, P_{tufB}, *mob*	Broad host range	Schleyer et al. (2008)
pEXGOX-A	Am^r, P_{tufB}, *mob*	Broad host range	Schleyer et al. (2008)
pEXGOX-K	Km^r, P_{tufB}, *mob*	Broad host range	Schleyer et al. (2008)
pZL1	Am^r, P_{tufB}	*G. oxydans* DSM2003	Zhang et al. (2010)
pBBR1p264	Km^r, $P_{gox0264}$, *mob*	Broad host range	Kallnik et al. (2010)
pBBR1p452	Km^r, $P_{gox0452}$, *mob*	Broad host range	Kallnik et al. (2010)
pBBR1MCS2_START	Km^r, P_{lac}, *mob*	Broad host range	Obranić et al. (2013)
pBBR1MCS3_START	Tc^r, P_{lac}, *mob*	Broad host range	Obranić et al. (2013)
pBBR1MCS4_START	Am^r, P_{lac}, *mob*	Broad host range	Obranić et al. (2013)
pBBR1MCS5_START	Gm^r, P_{lac}, *mob*	Broad host range	Obranić et al. (2013)
pBBR1MCS5*pgHp0169*	Gm^r, $P_{gHp0169}$, *mob*	Broad host range	Shi et al. (2014)

reduce cooling cost to control heat generated from fermentation reactions which would affect bacterial growth and production. Many thermotolerant *Acetobacter* strains with acetic acid resistance ability have been isolated from various sources around the world and they were proposed for industrial applications. On the other hand, *Gluconobacter* strains are known as sugar, sugar alcohol, and sugar acid oxidizers, producing a number of commercially valuable compounds. Many strains with features applicable for industrial production of these compounds have been isolated. Recently, genetic adaptation, as well as the development of genetic engineering tools for AAB, have become promising methods to improve the properties of AAB suitable for industrial applications.

Acknowledgments

The writing of this review was supported by grant LO1204 from the National Program of Sustainability I from the Ministry of Education, Youth and Sports, Czech Republic and by the project POST-UP II, reg. No. CZ.1.07/2.3.00/30.0041. J.F. and I.F. were partly supported by the Operational Program Education for Competitiveness — European Social Fund (project CZ.1.07/2.3.00/20.0165). Part of the work shown in the review was supported financially by the Program for Promoting Basic Research Activities for Innovative Biosciences (PROBRAIN) and the Advanced Low Carbon Technology Research and Development Program (ALCA) to K.M., and also by a Grant-in-Aid for Scientific Research (26450094) from the Ministry of Education, Science, Sports and Culture of Japan to O.A.

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Please cite this article as: Saichana N, et al, Acetic acid bacteria: A group of bacteria with versatile biotechnological applications, Biotechnol Adv (2014), http://dx.doi.org/10.1016/j.biotechadv.2014.12.001

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Please cite this article as: Saichana N, et al, Acetic acid bacteria: A group of bacteria with versatile biotechnological applications, Biotechnol Adv (2014), http://dx.doi.org/10.1016/j.biotechadv.2014.12.001